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Nucleotide sequence of the tryptophan decarboxylase gene of *Catharanthus roseus* and expression of *tdc-gus*A gene fusions in *Nicotiana tabacum*

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Abstract. The enzyme tryptophan decarboxylase (TDC; EC 4.1.1.28) converts tryptophan into tryptamine. In *Catharanthus roseus* and other plants capable of producing terpenoid indole alkaloids (TIAs) TDC links primary metabolism to the secondary metabolic pathway involved in the biosynthesis of these compounds. The accumulation of *tdc* mRNA in *C. roseus* cells is developmentally regulated and transcriptionally influenced by elicitors (induction) and auxins (repression). Here we report that TDC is encoded by a single copy gene in the *C. roseus* genome. No introns were observed upon isolation and sequencing of this gene. To study gene expression controlled by the *tdc* promoter, a 2 kb promoter fragment and a number of 5′ deleted promoter derivatives were joined in translational fusion to a β-D-glucuronidase reporter gene (*gusA*). Expression of the chimaeric constructs was monitored in stably transformed tobacco plants and in transiently transfected tobacco protoplasts. Histochemical and fluorimetric analysis of transgenic plants revealed that 1938 bp of the *tdc* promoter (with respect to the translational start codon) give rise to GUS activity in roots, stems and leaves. No tissue or cell type specificity was noted. Promoter deletions up to nucleotide −398 directed lower levels of *gusA* expression but conferred the same pattern of staining for GUS activity as the −1938 construct. Further deletion of the *tdc* promoter up to nucleotide −232 resulted in drastically reduced GUS activity levels and loss of GUS staining in all parts of the transgenic plants. In contrast to stable transformation, the −232 *tdc-gusA* construct gave rise to GUS activity levels comparable to those of the −398 construct in an assay system for transient expression in protoplasts. In this system the GUS activities measured were not affected by the presence or absence of the synthetic auxin naphthalene acetic acid (NAA).

Key words: *Catharanthus roseus* – Tryptophan decarboxylase – Gene expression – Nucleotide sequence – Promoter-*gus*A fusions

Introduction

In addition to primary metabolites essential for their growth and differentiation, plants produce a wide array of other compounds, known as secondary metabolites, with very diverse chemical structures. Although apparently non-essential for basic plant developmental processes, secondary metabolites generally contribute to the overall fitness of plants by providing protection against voracious animals and insects as well as pathogenic fungi, bacteria and viruses, or by attracting pollinating insects (Wink 1988; Phillipson 1990). Apart from these important ecological roles, plant secondary metabolites are also of commercial interest as valuable drugs, flavours, perfumes and pigments. The mechanisms which govern the activity of secondary metabolism in plants and derived cell cultures are poorly understood. Accumulation of secondary metabolites in plants is often limited to a certain organ or tissue type or to a particular stage in development. In cell cultures, secondary metabolite accumulation is usually incompatible with active cell proliferation and confined to the stationary phase of the growth cycle (Knobloch and Berlin 1980).

We have investigated the molecular basis of the expression of terpenoid indole alkaloid (TIA) biosynthesis in *Catharanthus roseus*. Some of the alkaloids formed in this pathway of secondary metabolism have important pharmaceutical applications. The root-derived monomeric alkaloids ajmalicine and serpentine are used in the treatment of circulatory diseases, while the leaf-derived dimeric alkaloids vincristine and vinblastine are used as

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anti-tumour agents. The enzyme tryptophan decarboxylase (TDC; EC 4.1.1.28) plays a key role in their biosynthesis as it links primary metabolism to TIA production by converting tryptophan into tryptamine. TDC was therefore selected as one of the targets for molecular biology studies.

A *tdc* cDNA clone was isolated from an expression library using antiserum raised against purified, denatured TDC (O. Goddijn 1992). Northern blot analysis revealed that *tdc* mRNA accumulation is extensively regulated. In cell suspension cultures, *tdc* mRNA accumulates after auxin starvation (Goddijn et al. 1992) and after exposure to fungal elicitors such as *Pythium aphidermatum* culture filtrates (Pasquali et al. 1992). In *C. roseus* seedlings and plants, *tdc* mRNA accumulation is also under developmental control (De Luca et al. 1988; Goddijn 1992). Here we report on the isolation and nucleotide sequence of the *tdc* gene. In a first attempt to understand its regulation, we have fused 5′ deletions of the promoter region to the β-D-glucuronidase reporter gene *(gusA)*. The expression of the different chimaeric constructs was tested in stably transformed tobacco plants and in transiently transfected tobacco protoplasts.

Materials and methods

Plant materials. Seeds of *C. roseus* (*Vinca rosea,* variety Morning Mist) were obtained from Kieft (Blokker, The Netherlands) and plants were grown in a greenhouse at 23° C with a photoperiod of 16 h. *Nicotiana tabacum* cv. Petit Havanna SR1 plants were grown in vitro at 27° C with a photoperiod of 12 h on 0.7% agar-solidified MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose.

DNA isolation. Total DNA was isolated from the top leaves of 2- to 3-month-old, non-flowering *C. roseus* plants according to the method of Mettler (1987) with some modifications. The leaves were frozen in liquid nitrogen and processed immediately or stored at −80° C. Frozen leaves were ground to a fine powder using a precooled mortar and pestle. Two volumes of homogenization buffer were added (1% Sarkosyl, 0.25 M sucrose, 50 mM NaCl, 20 mM Na_2EDTA, 50 mM TRIS-HCl pH 8.0) together with proteinase K to a final concentration of 50 μg/ml. The mixture was homogenized thoroughly and incubated at room temperature for 20 to 30 min. One volume of phenol (equilibrated with 0.5 M NaCl, 0.1 M TRIS-HCl pH 8.0) was added and mixed by gentle inversion for 3 min. Phases were separated by centrifugation in a Sorvall table centrifuge for 10 to 20 min at 2500 rpm. The aqueous phase was re-extracted once with one volume of phenol and once with one volume of chloroform. DNA was purified from the aqueous phase by CsCl gradient centrifugation according to Maniatis et al. (1982). The DNA obtained was then extensively dialysed against 10 mM TRIS-HCl pH 8.0, 1 mM Na_2EDTA (TE). DNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of 96% ethanol (−20° C). The DNA precipitate was collected on a glass rod, washed with 70% ethanol, dried, dissolved in TE and stored at 4° C.

Southern blot analysis. Ten micrograms of restriction enzyme-digested genomic DNA was electrophoresed in 0.7% agarose gels in TRIS-borate buffer (Maniatis et al. 1982) and blotted onto GeneScreen membranes (New England Nuclear) using 0.5 M NaOH as blotting solution. The *tdc* cDNA hybridization probe was prepared using the random primer labelling method (Feinberg and Vogelstein 1984). The membranes were prehybridized for several hours at 42° C in 50% deionized formamide, 5 × SSPE (1 × SSPE is 180 mM NaCl, 1 mM Na_2EDTA, 10 mM sodium phosphate pH 6.5) and 5% sodium dodecyl sulphate (SDS). Hybridization was performed for 24 h at 42° C in this prehybridization mixture supplemented with [α-^{32}P]dCTP-labelled *tdc* cDNA. After hybridization, the membranes were washed twice in 0.1 × SSPE, 0.1% SDS at 65° C for 15 min and exposed to Fuji-RX films mounted on Kyokko-LHII intensifying screens at −80° C.

Construction and screening of a genomic DNA library. Total genomic DNA (100 μg) was partially digested with *Sau*3AI and size fractionated by sucrose gradient centrifugation according to Ausubel et al. (1988). The fraction containing DNA fragments ranging from 9 to 13 kb in size was used to ligate to lambda GEM11 dephosphorylated *Bam*HI arms (Promega). Subsequent packaging (Promega packaging mix) resulted in a genomic DNA library. The phage titer was determined by transfection of various bacterial strains. The bacterial strain KW251 (Promega) gave rise to the highest transfection rate and subsequent platings were performed with this strain. About 425000 primary transformants, representing approximately seven times the size of the haploid *C. roseus* genome (6×10^8 bp as determined by cytoflow spectrophotometry; van der Have NV, Rilland Bad, The Netherlands), were screened by the plaque hybridization technique of Benton and Davis (1977) using nylon filters (Hybond-N, Amersham). Conditions for prehybridization, hybridization and washing were as described for Southern blot analysis.

Nucleotide sequencing. A genomic DNA fragment from λGEM clone A II containing the promoter region and part of the coding region of the *tdc* gene was subcloned as a *Bgl*II-*Eco*RI fragment in *Bam*HI + *Eco*RI-cut Bluescript plasmids KS and SK (Stratagene) to yield plasmids pGCR2-K and pGCR2-S. Deletion fragments of the insert were generated from the 5′ end in both plasmids using the Erase-a-Base system (Promega). Vector sequences were protected against *Exo*III nuclease activity by restriction with *Sac*I (in pGCR2-K) or *Kpn*I (in pGCR2-S). The resulting deletion fragments were sequenced by the dideoxy chain-termination method (Sanger et al. 1977). Double-stranded as well as single-stranded DNAs were used in the sequencing reactions. Single-stranded DNA was prepared from the Bluescript plasmids using helper phage R408 according to the Stra-

tagene sequencing protocol. Double-stranded DNA was isolated according to Maniatis et al. (1982); both strands of the *tdc* gene were sequenced. A genomic DNA fragment from λGEM clone A III containing the terminator region and part of the coding region of the *tdc* gene was subcloned as an *Xho*I fragment in the Bluescript SK vector. A genomic DNA fragment overlapping with both A II and A III and containing the *tdc* gene region missing in these fragments was isolated using the polymerase chain reaction (PCR) on total DNA of *C. roseus*. Both fragments were sequenced using synthetic primers. Primer extension analysis of *tdc* transcripts was performed according to Geliebter (1987). A primer was synthesized (5′-CGGCTATGAAATCTACC-3′), annealed to poly(A)$^+$ RNA isolated from *C. roseus* cell suspensions grown on induction medium (Knobloch and Berlin 1980) and extended in the presence of dideoxynucleotides. Sequence data were analysed using the University of Wisconsin Genetics Computing Group programs (Devereux et al. 1984).

*Construction of promoter-*gusA *fusions.* All DNA manipulations were essentially according to Maniatis et al. (1982). Progressively larger 5′ deletions of the *tdc* promoter generated in the pGCR2-K plasmid were subcloned as *Pvu*II-*Eco*RI fragments in the shuttle vector pIC 20H (cut with *Eco*RV and *Eco*RI). The promoter fragments were subsequently inserted as *Hin*dIII-*Bam*HI fragments into the binary plant vector pBI101.1 (Jefferson 1987), resulting in the plasmids pBTGUS A, C, D and E (Fig. 1). Using this strategy, a translational fusion between the *tdc* gene and the GUS coding sequence was created coding for a GUS protein which is N-terminally extended by 27 amino acids encoded by the *tdc* gene and 13 amino acids derived from pIC20H and pBI101.1. The final constructs were sequenced to confirm that the fusions were in-frame. Promoter-GUS fusions used in assays for transient expression were constructed in pUC8GUS.1 (S.T.C. Neuteboom, unpublished) using the same cloning strategy, resulting in the plasmids pUTGUS B, C, D and E. The pUC8GUS.1 plasmid contained the GUS coding region and flanking sequences from pBI101.1 cloned as a *Hin*dIII-*Eco*RI fragment in the *Hin*dIII-*Eco*RI sites of pUC8 (Vieira and Messing 1982).

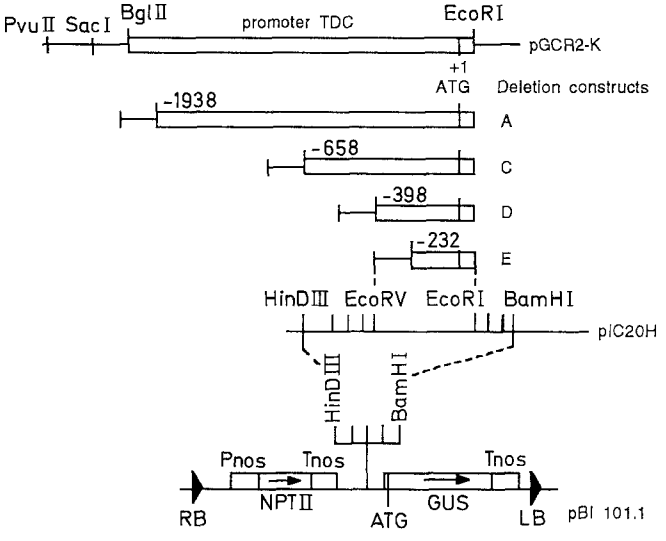

Fig. 1. Construction of fusions between the gene encoding tryptophan decarboxylase (*tdc*) and β-D-glucuronidase (GUS) reporter gene. The plasmids pBTGUS A, C, D and E were constructed by inserting the depicted promoter fragments in translational fusion with the GUS coding sequence of pBI101.1 via pIC20H (for details see text). Only the relevant restriction sites are shown. The *arrows* in the NPTII and GUS coding sequences indicate the direction of transcription. Pnos and Tnos, nopaline synthase promoter and terminator sequences; NPTII, neomycin phosphotransferase gene; RB and LB, right and left T-DNA border repeats

Plant transformation. The binary plant expression vectors pBTGUS A, C, D and E and the vector pBI101.1 were introduced into the disarmed *Agrobacterium tumefaciens* strain LBA4404 via electroporation (Mattonovich et al. 1989). The resulting transformed strains were used in *N. tabacum* cv. SR1 leaf disc transformation experiments according to Horsch et al. (1984). Selection of transformed shoots was on agar-solidified MS medium containing 100 μg/ml kanamycin and 100 μg/ml of each of the bacterial antibiotics vancomycin and cefotaxim. Shoots were rooted and subcultured by transferring stem segments harbouring axilliary buds to fresh selection medium without phytohormones.

Transient expression. Isolation of tobacco leaf mesophyll protoplasts from *N. tabacum* SR1 plants grown in vitro was performed essentially as described by Saul et al. (1988). Transformation of the protoplasts, using 10 μg plasmid DNA, 50 μg carrier DNA and 1×10^6 protoplasts, was performed according to the procedure of Pröls et al. (1988). After transformation, protoplasts were incubated in the dark at 27° C for 20 h.

Histochemical and fluorimetric assay of GUS activity. Histochemical localisation of GUS activity was performed essentially as described by Jefferson (1987) using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; Sigma) as substrate; staining was performed overnight at 37° C. Tissues were bleached by soaking in 70% ethanol and extracts were then prepared from tobacco tissues according to Jefferson (1987). Assays for β-D-glucuronidase activity were performed using 4-methyl umbelliferyl β-D-glucuronide (MUG; Sigma) as substrate. Samples were measured in a Perkin-Elmer fluorimeter using known concentrations of sodium-methylumbelliferone (MU; Sigma) to calibrate the instrument. Protein concentrations of the analysed samples were determined according to Bradford (1976) using bovine serum albumin as a standard.

Results

TDC is encoded by a single copy gene

To determine the number of *tdc* genes in the *C. roseus* genome, Southern blot analysis was performed. *C. roseus*

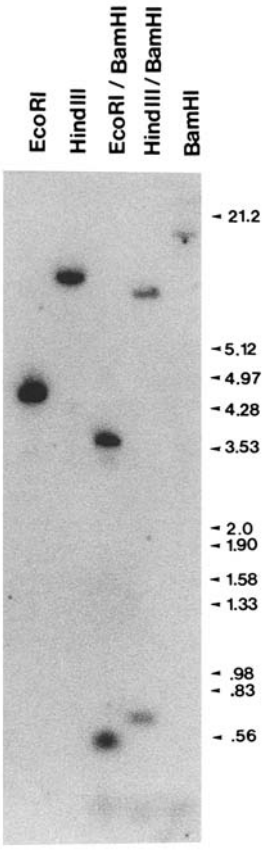

Fig. 2. Southern blot of *Catharanthus roseus* genomic DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII or combinations of these enzymes. The *tdc* cDNA clone (*Eco*RI-*Hin*dIII fragment) was used as probe. Positions and sizes in kb of DNA marker fragments are indicated

DNA was digested with three different restriction enzymes and hybridized under stringent conditions with a nearly full-length *tdc* cDNA clone (Fig. 2). In the case of digestion with *Eco*RI or *Hin*dIII only one hybridizing band was observed. Multiple hybridizing bands observed after *Bam*HI digestion and digestion with combinations of restriction enzymes can be explained by the occurrence of the corresponding restriction sites within the cDNA clone. These data demonstrated that TDC is encoded by a single gene in the *C. roseus* genome.

Isolation and sequence of the tdc *gene*

Screening of a primary *C. roseus* genomic DNA library with *tdc* cDNA as probe resulted in the isolation of two non-overlapping genomic clones, A II and A III. Restriction fragment analysis revealed that clone A II contained about 9 kb of upstream sequences and the 5′ part of the coding region of the *tdc* gene. Clone A III contained the 3′ part of the coding region and about 11 kb of downstream sequences. A *Bgl*II-*Eco*RI fragment from A II harbouring 2.4 kb of *tdc* promoter sequences including the translational start codon was subcloned in the Bluescript KS and SK vectors resulting in the plasmids pGCR2-K and pGCR2-S. In these plasmids, 5′ and 3′

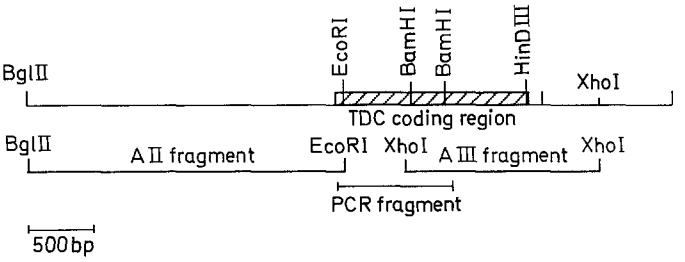

Fig. 3. Structure of the *tdc* gene and the fragments used for determining the nucleotide sequence

deletions were generated and used to sequence both strands of the *tdc* promoter region. The *tdc* coding sequence and 589 bp of the *tdc* terminator were determined by sequencing clone A III and a genomic DNA fragment overlapping with A II and A III obtained by PCR. The DNA fragments used for sequencing are shown in Fig. 3. Inspection of the nucleotide sequence (Fig. 4) revealed that the *tdc* gene does not contain introns. In accordance with the presence of only one *tdc* gene in the *C. roseus* genome, no differences were noted between the *tdc* cDNA sequence and the coding sequence of the *tdc* gene. Within the *tdc* promoter, several sequence motifs were found which have been reported to function as binding sites for plant transcription factors (see legend to Fig. 4).

Analysis of the 5′ end of tdc *mRNA*

To determine the 5′ end of *tdc* mRNA, primer extension was performed. As shown in Fig. 5, the RNA sequencing reaction was halted by a stretch of repeating CT residues resulting in a premature stop. Larger extension products were apparent in the sequencing lane without ddNTPs. The corresponding transcriptional start sites in the *tdc* gene are located approximately 24 nucleotides upstream of the CT box between positions −96 and −51 with respect to the translational start codon; a putative TATA box is found at position −153.

Expression of chimaeric tdc-gusA *gene constructs in transgenic* N. tabacum *plants*

To study gene expression driven by the *tdc* promoter, translational fusions were created between *tdc* promoter fragments and a β-glucuronidase *(gusA)* reporter gene. The fusion constructs were generated in the plant binary vector pBI101.1 using respectively, 1938 bp (A), 658 bp (C), 398 bp (D) and 232 bp (E) of the *tdc* promoter (relative to the ATG at position +1). *A. tumefaciens* strain LBA4404 was used to stably introduce these constructs into *N. tabacum* SR1 plants. Primary transformants were histochemically assayed for GUS activity in roots, stems (cross sections) and leaf segments. For each series of transformants ten kanamycin resistant plants were analysed. Transgenic plants obtained using LBA4404 containing the pBI101.1 vector showed no

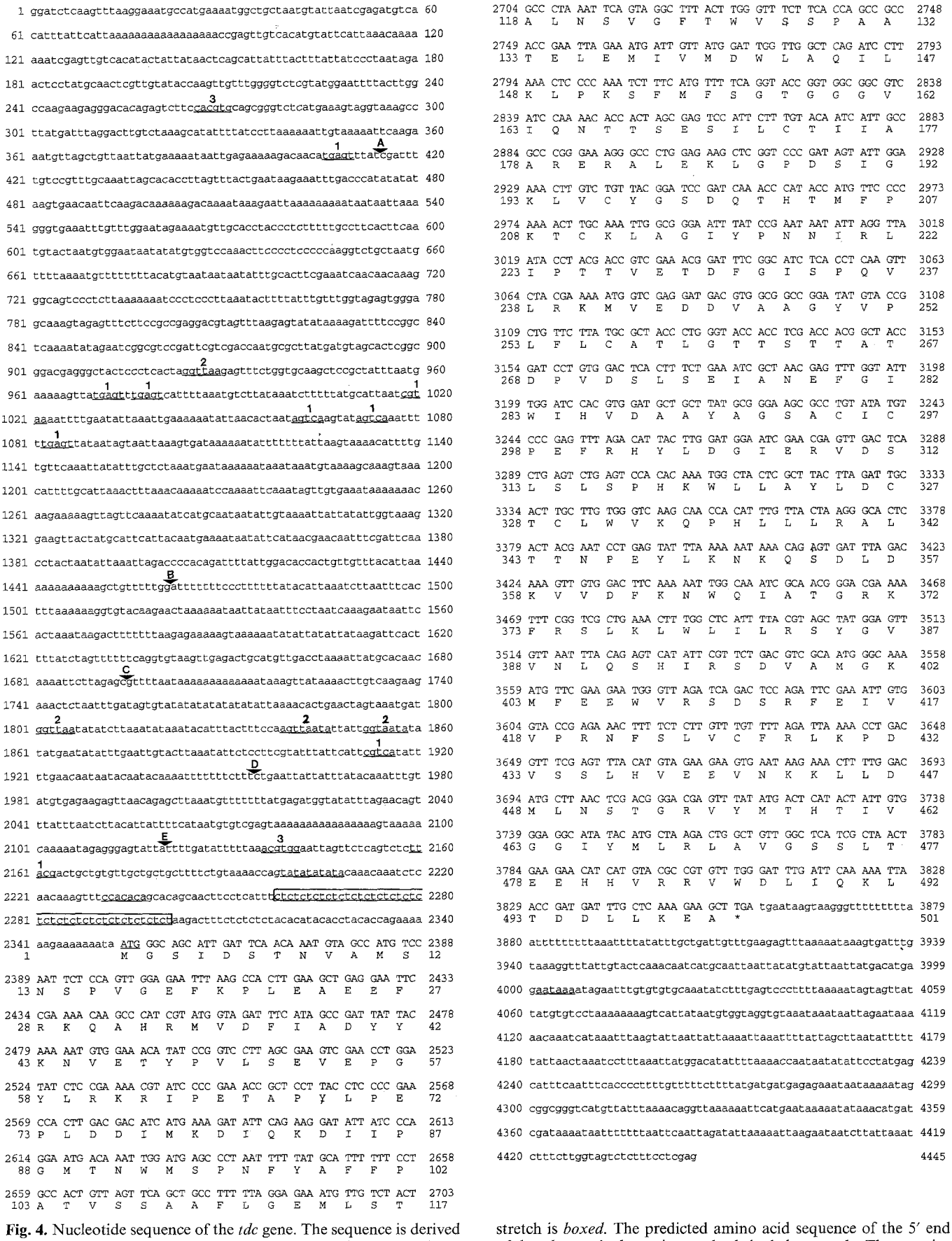

Fig. 4. Nucleotide sequence of the *tdc* gene. The sequence is derived from dideoxynucleotide sequencing of restriction fragments of two genomic clones and an overlapping genomic polymerase chain reaction (PCR) fragment (see the Materials and methods). The nucleotide sequence is labelled with the following letters shown in *boldtype:* **A** (position with respect to the ATG: −1938), **B** (−891), **C** (−658), **D** (−398) and **E** (−232) indicating the deletion endpoints used in the promoter studies. The putative TATA box (position 2199) and RNA start site (position 2231) are *underlined* A CT stretch is *boxed.* The predicted amino acid sequence of the 5′ end of the *tdc* gene is shown in standard single letter code. The putative polyadenylation site is *underlined* in the terminator. Sequences which may be involved in the binding of regulatory factors are *underlined and numbered:* 1, sequences which might be involved in the binding of ASF-1 (Lam et al. 1989); 2, putative binding site for GT-1 (Gilmartin et al. 1990); 3, G-box related sequence (Schulze-Lefert et al. 1989)

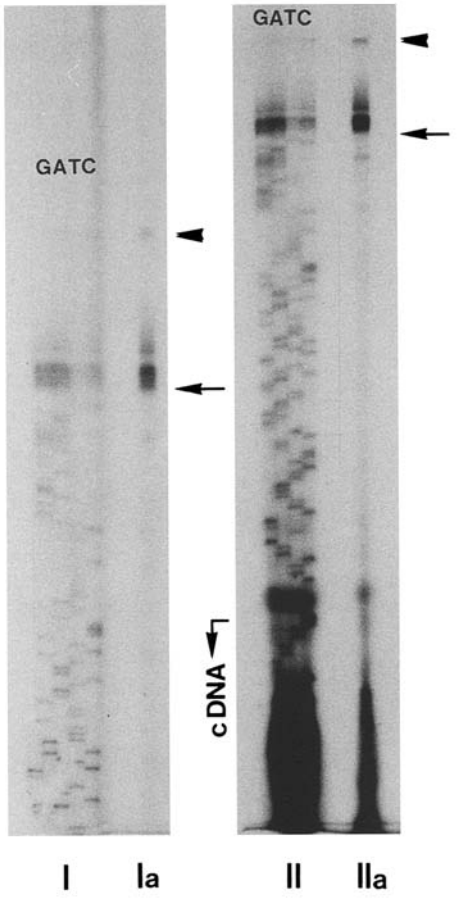

Fig. 5. Dideoxynucleotide sequencing of the 5′ end of *tdc* transcripts by primer extension. A sequencing primer was synthesized (5′-CGGCTATGAAATCTACC-3′) and annealed with poly(A)$^+$ RNA, which was isolated from *C. roseus* cell suspensions grown on induction medium (IM) and extended in the presence of ddGTP(G), ddATP(A), ddTTP(T) or ddCTP(C). The overlap with the *tdc* cDNA sequence is indicated. Lanes I and II display respectively a long and a short run of the sequence. Lanes Ia and IIa display sequencing reactions performed in the absence of dideoxynucleotides. The start of the CT box is indicated by an *arrow*. The putative RNA start sites are indicated by *arrowheads*

GUS activity in any of the examined tissues. Of ten plants resulting from transformation with the −1938 construct, six transformants showed activity in leaf, stem and root material; the intensity of staining, however, was highly variable. In leaf and stem material, activity was not confined to specific cell types. The GUS activity detected in roots was also not confined to specific regions (data not shown). In four transformants, no GUS activity at all was observed. Of the plants resulting from transformation with the −658 and −398 constructs, 5 and 7 plants, respectively, showed a staining pattern for GUS activity identical to that seen for the −1938 transformed plants although the intensity of staining was much lower. Of the plants transformed with the −658 and −398 constructs, 5 and 3 plants, respectively, showed no GUS staining in any of the tissues examined. The expression of *tdc-gusA* was even lower in plants resulting from transformation with the −232 construct: only one transformant displayed very weak staining in root material; all other transformants were negative.

Fluorimetric analysis of GUS activity was performed on leaf, stem and root material of the transgenic plants displaying GUS staining in the histochemical assay (Fig. 6). A random selection was made of plants transformed with the −232 construct. No GUS activity was detected in tissues from plants obtained by transformation with LBA4404 containing the pBI101.1 vector. The histochemical data obtained from all transformants correlated well with the GUS activities of the transgenes measured in the fluorimetric assay.

NAA does not affect the expression level of tdc-gusA *fusion constructs in protoplasts*

Chimaeric *tdc-gusA* genes were tested in an assay system for transient expression by transfecting tobacco leaf mesophyll protoplasts with constructs containing respectively 891, 658, 398 bp and 232 bp of the *tdc* promoter. The protoplasts were isolated and incubated in the presence or absence of 10^{-5} M of the synthetic auxin naphthalene acetic acid (NAA). The aim of this experiment was to see whether transcriptional down-regulation of the *tdc* gene, as observed in *C. roseus* cell suspension and hairy root cultures (Goddijn et al. 1992), could be assigned to certain regions within the *tdc* promoter. Analysis of the deletion series in the assay system revealed a gradual decrease in GUS activity levels as the promoter fragments decreased in length from 891 to 232 bp regardless of whether NAA was present or absent (Table 1). These findings indicate that, in contrast to the results for stable transformants, the 5′ *tdc* promoter fragments tested, even the shortest deleted to −232, are all active in tobacco mesophyll protoplasts.

Discussion

To enable studies on the molecular mechanisms governing expression of key biosynthetic genes in plant secondary metabolism, we have isolated a gene coding for the enzyme tryptophan decarboxylase of *C. roseus*. In this paper, we report the complete nucleotide sequence of the *tdc* gene. Furthermore, we describe the expression characteristics of a set of 5′ deleted *tdc* promoter fragments coupled to a *gusA* reporter gene and introduced into *N. tabacum*. Southern blot analysis indicated that the tryptophan decarboxylase enzyme is encoded by a single copy gene in the *C. roseus* genome. Inspection of the nucleotide sequence revealed that the *tdc* gene does not contain introns and, in accordance with the presence of a single copy of the gene, there are no differences between its coding region and the coding region of the *tdc* cDNA clone used as a probe in the isolation of the *tdc* gene. The single copy character of the *tdc* gene should facilitate studies on the molecular mechanisms that govern its expression, since differential expression of separate gene family members can now be excluded.

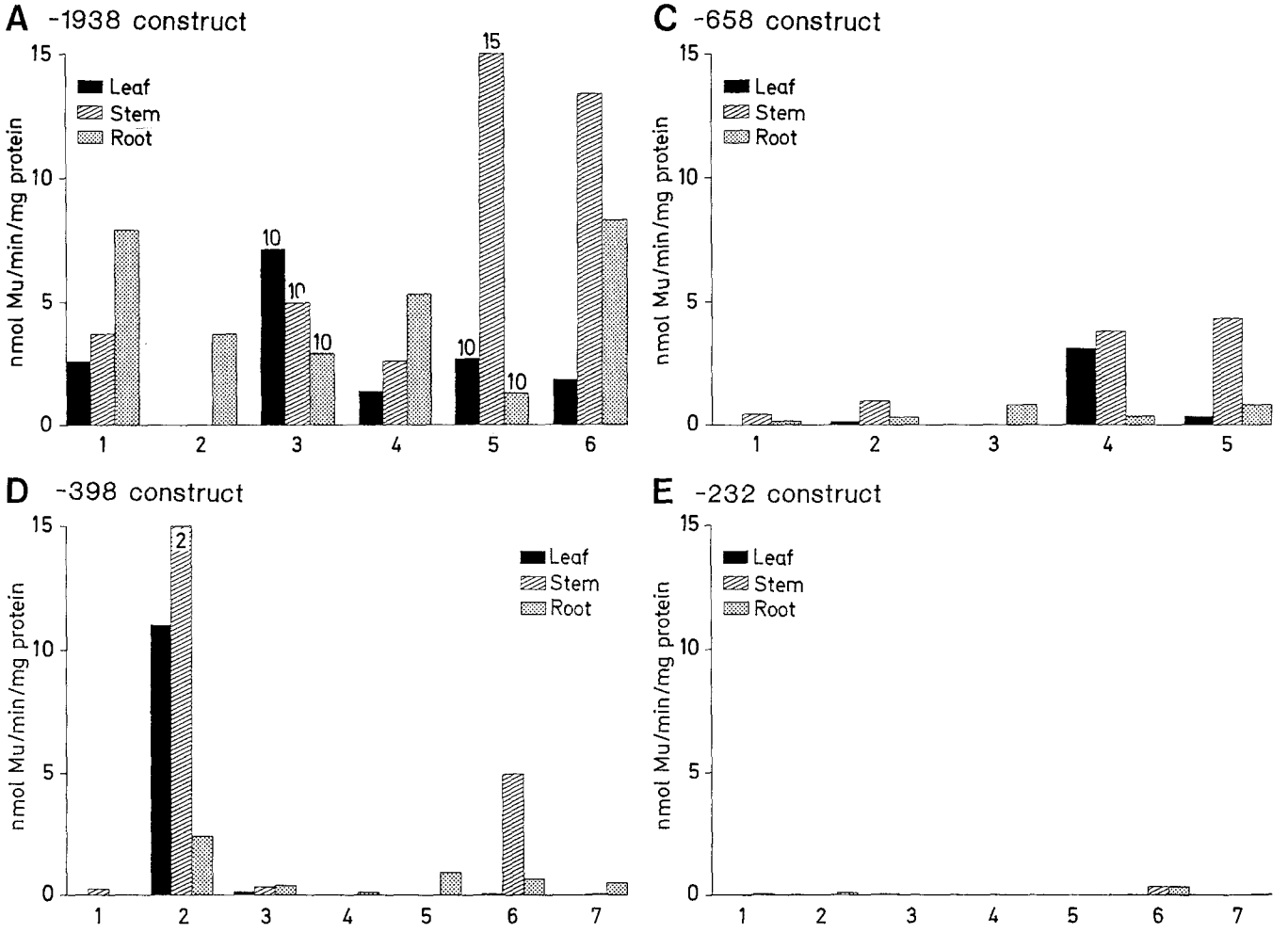

Fig. 6. Histograms showing quantitative analysis of GUS expression levels in transgenic *Nicotiana tabacum* plants containing pBTGUS A, C, D and E. If a *number* appears at the top of a bar, then the level of GUS activity should be multiplied by this factor. Mu, Sodium-methyl-umbelliferone

Table 1. Analysis of chimaeric *tdc*-GUS fusion constructs in an assay system for transient expression

Construct	Experiment I		Experiment II	
	−NAA	+NAA	−NAA	+NAA
−891 (B)	11.7	7.0	18.1	16.2
−658 (C)	8.9	7.2	16.6	12.6
−398 (D)	4.9	2.7	2.7	7.0
−232 (E)	2.4	3.8	6.2	3.8
Vector control	ND	ND	ND	ND

tdc, Gene encoding tryptophan decarboxylase; GUS, β-D-glucuronidase; NAA, naphthalene acetic acid; ND, not detectable
Values given are of GUS activity (nmol sodium-methyl-umbelliferone/min per mg protein) in tobacco leaf mesophyll protoplasts after transformation with the chimaeric *tdc*-GUS constructs B, C, D and E. Two separate experiments were performed (I and II) using protoplasts isolated in the presence (+) or absence (−) of 10^{-5} M NAA. GUS activity values are mean values of two measurements using the same protein extract

The expression characteristics of the *tdc* promoter were examined in stably transformed tobacco plants and in transiently transfected tobacco protoplasts. A number of 5′ deleted promoter fragments extending respectively from positions −1938, −658, −398 and −232 to +47 with respect to the translational start codon were linked to the *gus*A reporter gene to form translational fusions, and introduced into tobacco plants. Histochemical and fluorimetric analyses of GUS enzyme activity were performed in *in vitro* grown plants obtained after leaf disc transformation with the different constructs; the −1938 construct did not disclose any organ, tissue or cell type-specificity of the *tdc* promoter. No consistent differences were seen in GUS activities between leaf, stem and root material from independent transformants. Furthermore, the GUS activities directed by the *tdc* promoter were found to vary considerably between independent transformants. Such variations are probably due to differences in the position of chromosomal insertion and/or copy number of the *tdc-gus*A gene constructs in different plants.

The apparent lack of organ specificity of the *tdc* promoter in tobacco plants markedly contrasts with the

organ-dependent accumulation of *tdc* mRNA in *C. roseus* plants (Goddijn 1992). In greenhouse-grown as well as in *in vitro* cultured *C. roseus* plants, *tdc* mRNA levels are highest in roots, 5- to 10-fold lower in leaves and very low in stems. It should be emphasized, however, that the GUS activities detected in tobacco not only reflect transcription driven by the *tdc* promoter but also the stability and translational efficiency of the resulting *tdc/gusA* mRNA as well as the stability and enzymatic properties of the final *tdc/gusA* fusion protein. Similarly, differential *tdc* mRNA stability may contribute significantly to the preferential *tdc* mRNA accumulation in *C. roseus* roots. Another explanation for the observed GUS activity pattern in tobacco might be that a longer *tdc* promoter fragment is necessary to obtain preferential root expression of the *tdc-gusA* fusion gene. Alternatively, the preferential *tdc* mRNA accumulation in *C. roseus* roots could be due to the presence of one or more transcription factors that are absent in tobacco. Transformation of *N. tabacum* plants with the complete *tdc* gene from *C. roseus* could provide clues as to which of these possibilities applies.

Analysis of the leaf, stem and root material of the plants obtained by transformation with the −658 and −398 constructs revealed lower GUS activities compared to plants transformed with the −1983 construct. This suggests that enhancer elements are located between positions −1938 and −658 which augment *tdc* promoter activity. Further deletion of the *tdc* promoter up to position −232 almost completely abolished *tdc*-directed GUS activity. Elements required for basal activity of the *tdc* promoter in a heterologous plant expression system may therefore be located between positions −398 and −232. Based on sequence homologies, a number of sequence motifs in the *tdc* promoter were marked (see Fig. 4) which represent possible target sequences for specific transcription factors. Our data, however, do not allow us to make any predictions concerning the functionality of these sequences in the *tdc* promoter.

The chimaeric *tdc-gusA* constructs were also used to study transient expression in tobacco protoplasts in order to identify sequences which are involved in the down-regulation of the *tdc* gene by auxin. In contrast to the down-regulation of *tdc* mRNA in *C. roseus* cell suspensions and hairy root cultures (Goddijn et al. 1992), protoplasts did not show any significant difference in GUS activity in the presence of NAA after transfection with the fusion constructs. Unexpectedly, transfection of protoplasts with the −232 construct resulted in GUS activity levels in the same range as found for the −398 construct. This is in striking contrast to the expression in stably transformed plants, where the −232 construct did not give rise to any substantial GUS activity. Apparently in the protoplast condition there is a positive effect on the expression of the −232 chimaeric construct. It is conceivable that this may be caused through an elicitor effect. The −232 chimaeric construct may still contain sequences which are instrumental in gene activation via elicitors. As shown in our laboratory, *tdc* mRNA is transcriptionally induced in *C. roseus* suspension cultures after treatment with elicitors (Pasquali et al. 1992). Data were recently obtained showing induction of GUS activity after treatment of leaf discs of −232*tdc-gusA* transgenic plants with elicitors (P.B.F. Ouwerkerk, personal communication). The generation of protoplasts gives rise to cell wall fragments which are known to elicit genes in protoplasts and perhaps also transient expression of introduced genes. Further analysis of the chimaeric constructs in transgenic *C. roseus* and *N. tabacum* cell suspensions will be required to identify sequences involved in the regulation of *tdc* by auxin and elicitors.

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